Advancing systems medicine based methods to predict drug response in diabetic kidney disease

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Chapter 5

A metabolomics based molecular pathway analysis for how the SGLT2-inhibitor dapagliflozin may slow kidney function decline in patients with diabetes

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Abstract

Background: Sodium glucose cotransporter-2 inhibitors (SGLT-2i) slow progression of diabetic kidney disease (DKD). The underlying mechanisms are not fully elucidated. We examined which metabolic pathways are targeted by the SGLT-2i dapagliflozin to explore the molecular processes involved in the renal protective effects.

Methods: An unbiased MS plasma metabolomics assay was performed on baseline and follow-up (week 12) samples from the EFFECT II trial in type 2 diabetes patients with non-alcoholic fatty liver disease receiving dapagliflozin 10 mg/day (n=19) or placebo (n=6) Transcriptomic signatures from tubular compartments were identified from kidney biopsies collected from patients with DKD (n=17) and healthy controls (n=30) from the European Renal cDNA Biobank (ERCB). Serum metabolites that significantly changed after 12 weeks dapagliflozin were mapped to a metabolite-protein interaction network. These proteins were then linked with intra-renal transcripts that were associated with DKD or eGFR. The impacted metabolites and their protein coding transcripts were analyzed for enriched pathways.

Results: Of all measured (n=812) metabolites, 108 changed ($p<0.05$) during dapagliflozin treatment and 74 could be linked to 367 unique proteins/genes. Intra-renal mRNA expression analysis of the genes encoding the metabolite-associated proteins using kidney biopsies resulted in 105 genes that were significantly associated with eGFR in patients with DKD, and 135 genes that were differentially expressed between patients with DKD and controls. The combination of metabolites and transcripts identified four enriched pathways that were affected by dapagliflozin and associated with eGFR: Glycine Degradation [mitochondrial function]; TCA Cycle II [energy metabolism]; L-carnitine Biosynthesis [energy metabolism] and Superpathway of Citrulline Metabolism [nitric oxide synthase and endothelial function].

Conclusion: The observed molecular pathways targeted by dapagliflozin and associated with DKD suggest that modifying molecular processes related to energy metabolism, mitochondrial function, and endothelial function may contribute to its renal protective effect.

Introduction

Sodium-glucose cotransporter 2 inhibitors (SGLT2i) are approved for glucose lowering treatment of type 2 diabetes patients. SGLT2i show beneficial effects on body weight, blood pressure and albuminuria and reduce the risk for heart failure hospitalization and cardiovascular (CV) death as well as end-stage renal disease in patients with diabetic kidney disease.[1–4] The precise underlying mechanisms responsible for these protective effects remain to be resolved. Although SGLT2is are primarily indicated as glucose lowering agents, they have a broad range of effects that could explain the salutary effects on CV and renal health including effects on energy metabolism, renal function, electrolyte and plasma volume homeostasis.[5–8]

Although several theories on the mechanism of action have been put forward, sophisticated mechanistic studies in humans as well as unbiased approaches are needed to understand the molecular mechanism underlying the protective effect of SGLT2i on CV and renal outcomes. To this end, a prior study performed untargeted metabolomics, i.e. the measurement of low-weight intermediates and end-products of cellular functions in biological fluids, to investigate the effect of short term (4 weeks) treatment with the SGLT2i empagliflozin on serum metabolites in patients with type 2 diabetes.[9] However, this study only described a subset of metabolites changed during empagliflozin treatment and did not perform an integrative bio-informatic approach to examine which kidney tissue associated molecular pathways may be associated with SGLT2i-altered metabolites.

In the current study we have used an unbiased high-throughput metabolomics assay to measure metabolites involved in all major metabolic pathways in plasma from patients with type 2 diabetes treated with dapagliflozin for 12 weeks. We subsequently integrated the metabolites with transcriptomic features measured in kidney tissues in a bio-informatic analysis to identify molecular pathways for how dapagliflozin may exert renal protective effects to improve our understanding of the mechanistic action of dapagliflozin.

Materials and Methods

Study design

For the current study, all type 2 diabetes patients treated with dapagliflozin (n=19) were selected from the EFFECT II study.[10] The design and primary results of the EFFECT II study were published previously.[10] In short, it was a 12-week multicenter randomized placebo-controlled double-blind four-arm parallel-group trial performed at five clinical

research centers at university hospitals in Sweden. Individuals enrolled in this study were eligible if they had been treated with a stable dose of metformin or sulfonylurea alone or in combination for at least 3 months, if they had a liver proton density fat fraction (PDFF) >5.5%, which is commonly used as a cut-off for Non-Alcoholic Liver Fatty Disease (NAFLD), and a body mass index >25 kg/m^2. Exclusion criteria included use of SGLTis, insulin or glucagon-like peptide receptor agonists, history of hepatic disease and creatinine clearance <60ml/min (Cockcroft-Gault). Patients were randomly assigned to dapagliflozin 10 mg/day, omega-3 carboxylic acids 4g/day, combined dapagliflozin and omega-3 carboxylic acids, or matching placebo. For the purpose of this analysis, data from patients with available samples for metabolomics analysis and assigned to dapagliflozin (N=19) or placebo (N=6) was used. The study was approved by the Regional Ethics Review Board in Uppsala, registered at ClinicalTrial.gov (ClinicalTrial.gov identifier NCT02279407) and conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonisation of Good Clinical Practice. All participants provided written informed consent before participating.

To link the metabolomics signature derived from the EFFECT II study to a kidney specific pathophysiological context, we used cross-sectional transcriptomics data from patients with diabetic kidney disease (DKD) (N=17) and healthy donors (N=30) who participated in the European Renal cDNA Bank-Kroener-Fresenius Biopsy Bank (ERCB) cohort (N=47). The design and characteristics of these participants have been described previously. [11,12]

Participant characteristics and measurements

In the EFFECT II cohort, patient characteristics were obtained as previously described.[10] In brief, height and weight were measured with standard methods in light clothing without shoes and BMI calculated as kg body weight divided by height (m) squared. Fasting blood samples were taken in the morning before intake of the investigational products. HbA1c was determined with ion-exchange high-performance liquid chromatography (All Variant II and Variant II Turbo Hemoglobin A1c reagents, Bio-Rad, Hercules, CA, USA).

In the ERCB cohort, fresh renal biopsy samples from patients with DKD and healthy donors were micro-dissected into glomerular and tubulointerstitial compartments. RNA was isolated and prepared for microarray analysis as described previously[11,12].

Table 1. Baseline characteristics from the EFFECTII and ERCB cohorts

	EFFECT-II		ERCBǂ	
	Dapagliflozin (n=19)	Placebo (n=6)	Living Donor (n=30)	Diabetic Kidney Disease (n=17)
Age, years	64.7 (6.6)	64.7 (6.9)	48 (12)	58 (10)
Sex				
Male	14	3	15	12
Female	5	3	15	5
BMI, kg/m^2	30.5 (2.9)	30.7 (2.2)		
Diabetes duration, years	4.7 (9.3)	7.2 (7.0)		
HbA1c, %	7.3 (0.5)	7.9 (0.6)		
Cholesterol, mmol/L	4.9 (1.0)	4.2 (1.2)		
Triglycerides, mmol/L	2.1 (1.2)	2.2 (1.0)		
Diastolic blood pressure, mmHg	86.2 (7.8)	84.6 (6.2)		
Systolic blood pressure, mmHg	147.3 (12.2)	136.2 (6.7)		
eGFR, mL/min per 1.73^2	86.5 (11.2)	87.7 (11.9)	106.2 (30.9)	44.3 (24.9)
Diabetes medication				
Metformin, n (%)	11 (58%)	4 (67%)		
Sulfonylurea, n (%)	1 (5%)	0 (0%)		
Metformin + Sulfonylurea, n (%)	4 (20%)	1 (17%)		
None/Other, n (%)	3 (16%)	1 (17%)		
Hypertension medication				
ACEi, n (%)	8 (42%)	1 (17%)		
ARB, n (%)	6 (32%)	3 (50%)		

ǂ Clinical chemistry and diabetes medications were not recorded in the ERCB cohort

Metabolomic platform

The non-targeted metabolomic analysis was performed at Metabolon, Inc (Morrisville, NC). All plasma samples were stored at -80°C until processed. Fasting plasma samples from baseline (visit 1) and end-of study (visit 4)were extracted. The samples were extracted with methanol and the supernatants divided into five equal fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI) optimized for more hydrophilic and hydrophobic compounds respectively, one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by hydrophilic interaction liquid chromatography//UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup [13] All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The MS analysis alternated between MS and data-dependent MS^n scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z. Compounds were identified by comparison to standard library entries of purified standards or recurrent unknown entities based on retention time, molecular weight, preferred adducts and in-source fragments, as well as associated MS spectra and curated by visual inspection for quality control using proprietary software developed by Metabolon®. The method for which each metabolite is quantitated is dependent on factors such as interference by neighboring peaks and reproducibility/variability. The quantitation was performed by proprietary software which matches ions to an in-house library of standards for metabolite identification followed by metabolite quantitation by peak area integration. [14]

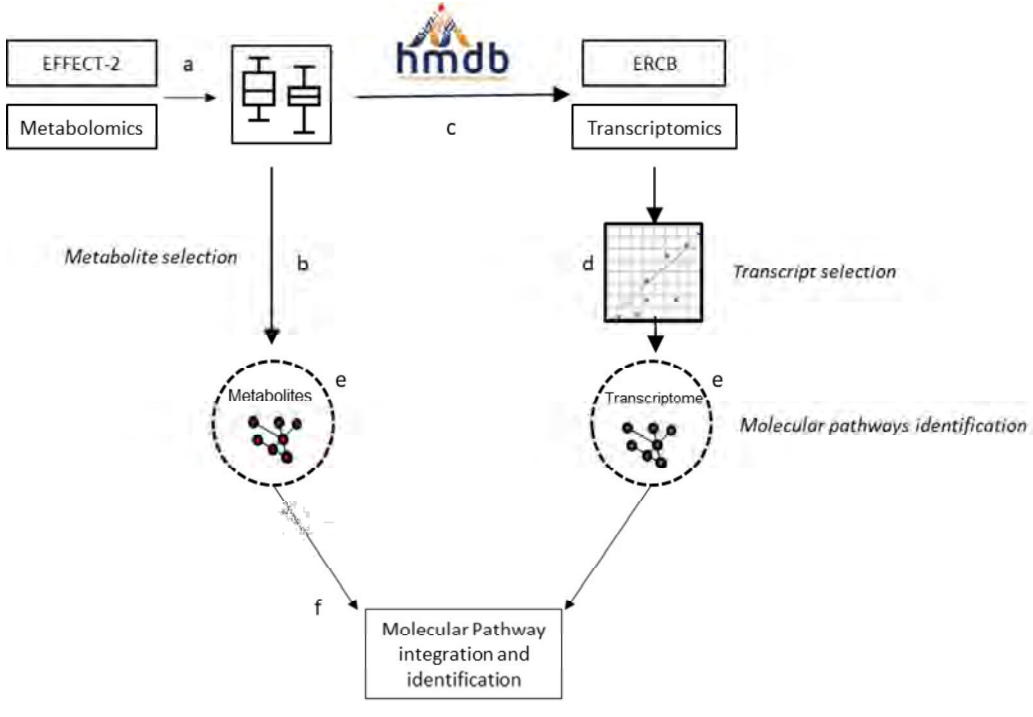
Statistical Analysis

Before analysis the metabolic dataset was imputed with the lowest observed value in the sample for that metabolite. All values were median scaled and before analysis transformed using the natural logarithm. Changes in metabolites were primarily evaluated in a univariate fashion. To determine whether metabolites had significantly changed, paired t-tests were used on the difference between baseline and end of study in logarithmic scale. The results of the paired t-tests are presented as geometric mean ratios (GMR) and p-values. No adjustment for multiplicity was performed because of the hypothesis generating nature of study, instead we evaluated the results based on a pathophysiological context and pathway analysis using the significant changes from the paired t-test. A p-value below 0.05 was regarded as significant he

changes in metabolites were also adjusted for changes in fasting plasma glucose by looking at the intercept of a linear regression model with change in the logarithm of glucose as a covariate. In an additional analysis, the effect of dapagliflozin compared to placebo on the change in the metabolite from baseline was also assessed by an analysis of covariance model (ANCOVA) with treatment and baseline measurement as covariates. Due to the small sample size of the placebo group a p-value ≤ 0.1 was adopted to indicate statistical significance.A sensitivity analysis was performed adding an interaction term in the ANCOVA. All statistical analyses were performed with R version 3.3.3 to 3.5.2 (R Project for Statistical Computing, www.r-project.org). A schematic overview of the multi-omics approach to identify molecular pathways associated with progressive kidney function decline is shown in Figure 1. All metabolomic features were used for analyses as there were no missing values. The human metabolite database (HMDB) version 4.0 was used to select related genes. Significant metabolites ($p \leq 0.05$) identifiable by a human metabolome database (HMDB) identifier were used for mapping and pathway analysis. To link these metabolomic molecular features to a kidney-specific pathophysiological context, all significant metabolomic molecular features that could be mapped to a gene were subsequently associated with kidney biopsy derived transcriptomic features. SGLT2i induced changes in metabolome are likely affecting the high energy demanding tubules making it a likely candidate for cross-omics integration. To select the transcripts, we used the annotated protein-metabolite interactions in the HMDB and selected protein coding transcripts. The selected transcriptomic features were used for the pathway integration if they either correlated with estimated glomerular filtration rate (eGFR) assessed by Pearson correlation in patients with DKD, or when they were differentially expressed between healthy living donors without diabetes or chronic kidney disease and patients with DKD, as assessed by student t-test (Figure 1).

Ingenuity pathway analysis (IPA) (QIAGEN) version 01-14 software was used to assign enriched pathways from the selected molecular metabolic or transcriptomic features using a Fischers exact test. The significantly enriched pathways ($p < 0.05$) based on transcriptomic or metabolomics features were compared. The set pathways identified by overlapping significant pathways from both metabolites and transcripts were used as a likely mechanism on a mechanistic molecular level how dapagliflozin can attenuate kidney function decline.

Figure 1: Schematic overview of a metabolomics to intra-renal transcriptomics approach to identify molecular pathways targeted by dapagliflozin and associated with progressive kidney function decline. Metabolomics were performed in the EFFECT-II randomized controlled trial (a). Metabolites changed during dapagliflozin were identified (b). To link the metabolomic features with kidney-specific pathophysiology context, unique protein coding genes derived from metabolomic features that significantly changed during dapagliflozin treatment were identified (c), and the gene expression profiles measured in kidney *tissues* from ERCB participants representing these genes were selected. The gene expressions were then associated with eGFR decline, and significant features were selected (d). Pathway analysis was then performed based on selected on metabolomics and transcriptomic features (e), and integration analysis of enriched molecular pathways based on metabolites and intra-renal transcripts was performed to select molecular pathways targeted by dapagliflozin and associated with DKD progression (f).



Results

Participants

The participants in the EFFECT II study had an average age of 64.7 years and were all overweight or obese (Table 1). At baseline, the mean diabetes duration was 4.7 years. Most individuals were treated with metformin alone (58%) or in combination with sulfonylurea (21%) and 16% were drug naïve and one patient were treated with sulfonylurea alone (5%). No change in medication occurred during the study period. Mean HbA_{1c} was 56 mmol/mol or 7.3%. As previously reported, dapagliflozin treatment decreased body weight 3% ($p<0.05$) and fasting glucose 10% ($p<0.05$) from baseline.[9] In the ERCB cohort participants with DKD had a lower eGFR compared to living donors and EFFECT II participants (Table 1).

Metabolomic profiles

A total of 1216 metabolites were measured including 812 identified metabolites and 404 unknown. The paired t-test evaluating differences between baseline and week 12 in the dapagliflozin treatment arm identified 108 metabolites that significantly changed, of which 22 metabolites were significantly increased and 86 were significantly reduced (Supplement table 1 and Supplement table 2). In order to get an understanding on how the reduction of glucose could have influenced the metabolites, the changes in metabolites were also adjusted for changes in glucose (Supplement table 1 and 2). An additional analysis was performed to examine the effect of dapagliflozin compared to placebo on the metabolites. Fourteen metabolites increased compared to placebo while 16 metabolites decreased compared to placebo (Supplementary table 1 and 2).

The subsequent presentation and analyses are based on the placebo unadjusted metabolite changes during dapagliflozin treatment. Metabolites belonging to the super pathway of amino acids were largely overrepresented among the metabolites significantly increased and constituted more than 80% of the identified metabolites, while the remaining metabolites belonged to the super pathways of xenobiotics, carbohydrates and co-factors and vitamins (Supplement Table 1). The most significant was N-acetyl aspartate (NAA) that is uniquely synthesized by neuronal mitochondria.[15] An increase in carnitines derived from partly metabolized BCAAs (isobutyryl-, isovaleryl- and tiglylcarnitines) was observed, while the levels of the corresponding amino acids did not change. Three out of the 22 metabolites identified as significantly increased by SGLT2i belonged to the sub pathway of histidine metabolism. Plasma levels of urea as well as 4 other metabolites, including N6, N6, N6-

trimethylysine and arginate, belonging to the urea cycle sub pathway were increased. Plasma levels of creatine, sarcosine, and heme also increased.

Among the metabolites that were reduced, lipids were overrepresented and constituted >50% of the down-regulated identified metabolites (Supplement table 2). Lipid species from several different lipid classes were reduced, including diacylglycerols, endocannabinoids, dicarboxylic acids and monohydroxylated, mainly 3-hydroxylated fatty acids. A marked reduction in a dihydroxy fatty acid, 12,13-HOME, was also observed. Interestingly, several primary and secondary bile acids were reduced indicating reduced bile acid pool. As expected, treatment reduced plasma glucose levels and other simple carbohydrates. The change in fasting glucose correlated well with the quantitative changes in fasting glucose measured previously (ρ=0.79, $p<0.0001$). As previously observed, plasma levels of urate were reduced.[5] Three metabolites in xanthine metabolism including theophylline, which is upstream urate in purine degradation, were reduced. Alanine and glutamine were the only amino acids reduced in plasma. Intermediates in the TCA cycle, including succinate, fumarate and malate, were reduced.

Metabolomics to transcriptomics linkage

As a next step, we took advantage of our database containing transcriptomic profiles derived from micro-dissected tubulointerstitial compartments of patients' biopsies and corresponding eGFR. The aim was to identify early effects of dapagliflozin on kidney function by correlating changes associated with DKD to effects of dapagliflozin in the diabetic EFFECT II patients with normal eGFR. To link the metabolites to a kidney-specific pathophysiological context, the metabolomic molecular features that significantly changed during dapagliflozin treatment and could be linked to a gene were subsequently associated with kidney biopsy derived transcriptomic features. The number of metabolites and transcripts that were selected for pathway identification are shown in table 2. Of the 812 measured metabolites 535 metabolites could be linked to a known HMDB identifier. Of these identified metabolites, 74 (Supplement Table 1and 2) were significantly changed by dapagliflozin and were mapped to proteins. Since a single metabolite can be derived from multiple proteins, the 74 known metabolites resulted in 367 unique proteins with corresponding protein coding gene (Table 2). To link these proteins to molecular signatures within a kidney-specific context, genes were selected from the same proteins that were associated with the metabolomics molecular features that changed upon dapagliflozin treatment. The expression value of these genes was then extracted from transcriptomic data derived from micro-dissected

tubulointerstitial compartments of patients' biopsies. Of the 367 selected tubular protein coding genes, 292 passed quality control. Linear regression analyses and differential expression analysis subsequently showed that 105 genes were significantly associated ($p<0.05$) with eGFR in patients with DKD (n=17) and 135 genes were differentially expressed ($p<0.05$) between DKD (n=17) and healthy donors (n=30) (Table 2).

Table 2: Summary of feature selection. The number of features measured and associated with drug and DKD are shown in the table for the omics and stratified by disease stage.

	Features measured (N)*	Features selected (N)‡	Unique Compounds (N)†	Unique protein coding genes
Metabolomics				
SGLT2	812	108	74	367
Transcriptomics				
Tubular cross sectional eGFR	292	105	105	105
Tubular DN vs healthy	292	135	135	135

* Number features that could be measured using the assay

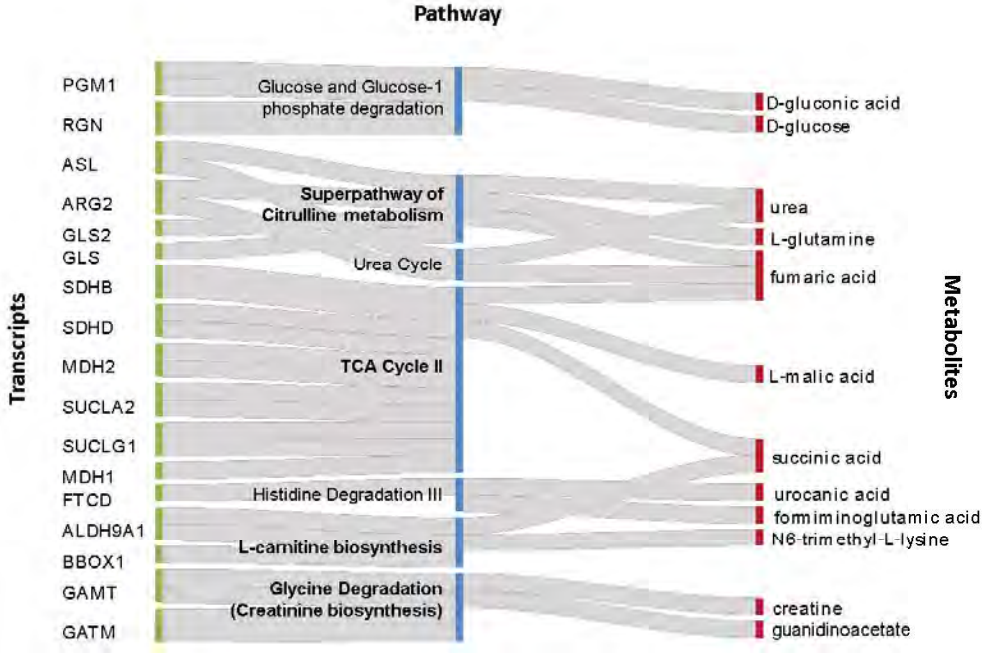
‡ Identifiable features by univariate analysis or machine learning

† Unique identifiable features by univariate analysis or machine learning

Pathway analysis

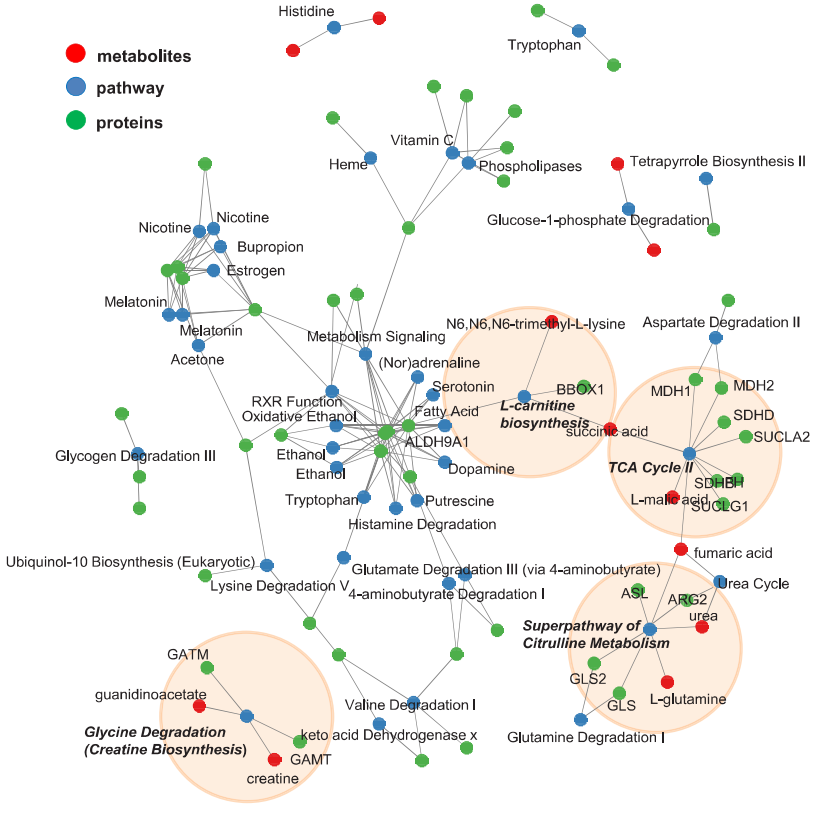
Pathway selection occurred on the basis of the overlap between the enriched pathways derived from selected transcriptomic and metabolomics features. At the pathway level, enrichment analysis of the significantly deregulated metabolites resulted in 7 metabolic pathways (Figure 2). Four of these pathways were also significantly enriched in the transcriptome and resulted in 15 unique genes and 11 metabolites of interest (Table 3, Figure 3). Thus, in total, 4 molecular pathways associated with dapagliflozin and diabetic kidney disease were identified based on the overlap between metabolomic and transcriptomic derived pathways (Figure 3). The bio-informatic analysis revealed that dapagliflozin upregulated the superpathway of citrulline metabolism, as well as the TCA cycle II, and the L-carnitine biosynthesis pathways and seemed to decrease the glycine degradation (creatine biosynthesis) pathway. When comparing dapagliflozin with placebo treatment, metabolites included in the superpathway of Citrulline Metabolism and the glycine degradation pathway were significantly different, while metabolites in the TCA cycle II and L-carnitine pathways were not significantly different between the groups.

Figure 2: Pathways significantly enriched in features based on metabolites affected by dapagliflozin.



Significant genes (green, left column) derived from the renal tissue transcriptomics and associated with eGFR or significantly different between patients with diabetic kidney disease and healthy donors are shown. Metabolites which significantly changed during dapagliflozin and represented in the enriched pathways are shown in red on the right side of the figure. In the middle, enriched pathways based on the metabolites are shown in blue, with the bold pathways also having significant enrichment in the kidney transcriptome.

Figure 3: Identified molecular pathway based on metabolite and intra-renal transcripts integration.



Molecular pathways highlighted in light orange indicate pathways targeted by dapagliflozin and associated with DKD progression.

Table 3: Summary of pathways and their respective mapping across omics and features sorted by p-value of the univariate enrichment analysis.

Pathway*	Metabolites changed during dapagliflozin treatment	Intra-renal transcripts and correlation with eGFR in DKD	Difference in transcripts between DKD and healthy control
Superpathway of Citrulline Metabolism	fumaric acid, L-glutamine, urea	GLS2, ASL, GLS	ARG2,
TCA Cycle II	succinic acid, fumaric acid, L-malic acid	SDHB,FH,SDHD,MDH2, SUCLA2,MDH1,SUCLG1	SDHB, FH, SDHD, MDH2, SUCLA2, SUCLG1
Glycine Degradation (Creatine Biosynthesis)	creatine, guanidinoacetate	GAMT, GATM	GAMT, GATM
L-carnitine Biosynthesis	N6,N6,N6-trimethyl-L-lysine, succinic acid	BBOX1, ALDH9A1	ALDH9A1

P values from Fisher exact test for enrichment of each pathway by metabolites were 0.014, 0.016 0.020 and 0.028 for superpathways of citrulline metabolism, TCA cycle II, glycine degradataion and L-carnitine biosynthesis, respectively. P-values for enrichment of these pathways by transcripts were 0.039, <0.01, 0.014, 0.023, respectively. *Abbreviations:* **GLS2** glutaminase 2 (liver, mitochondrial), **ASL** argininosuccinate lyase, **GLS,** glutaminase; **ARG2,** arginase 2; **SDHB,** succinate dehydrogenase complex, subunit B, iron sulfur (Ip); **FH,** fumarate hydratase; **SDHD,** succinate dehydrogenase complex subunit D integral membrane protein; **MDH2,** malate dehydrogenase 2 NAD (mitochondrial); **SUCLA2,** succinate-CoA ligase ADP-forming beta subunit; **MDH1,** malate dehydrogenase 1 NAD (soluble); **SUCLG1** succinate-CoA ligase alpha subunit; **GAMT,** guanidinoacetate N-methyltransferase; **GATM,** glycine amidinotransferase (L-arginine:glycine amidinotransferase); **BBOX1,** butyrobetaine (gamma) 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1; **ALDH9A1**, aldehyde dehydrogenase 9 family member A1

Discussion

In this study, we investigated the effect of 12 weeks of treatment of dapagliflozin, an SGLT2 inhibitor, on plasma levels of more than 800 identified metabolites. In general, our results show that treatment of dapagliflozin in the fasting situation is associated with increased amino acid metabolites and reduced levels of lipid species from several subclasses. We used a bio-informatic approach to delineate molecular pathways that may contribute to the renal protective effects of the SGLT2 inhibitor dapagliflozin. Overlap at the gene expression level between metabolites targeted by dapagliflozin and intra-renal transcripts associated with DKD pointed to upregulation of superpathway of citrulline metabolism, the TCA cycle II, and L-carnitine biosynthesis as well as possibly a down-regulation of the glycine degradation pathway (creatine biosynthesis). These molecular pathways are linked to energy metabolism, mitochondrial function and endothelial function suggesting that dapagliflozin treatment may slow progression of DKD by altering activity of these pathways.

In line with previously published metabolomics results based on one month treatment with empagliflozin several of the increased metabolites belonged to the sub pathway of BCAA, while the corresponding BCAAs were not changed.[9] Also, urea and urea cycle metabolites, including N2, N5 diacetylornithine, N-delta-acetylornithine, and argininate were similarly increased in the two studies. In contrast to the findings by Kappel et. al.[9], we observed decreased levels of TCA metabolites. The reason for the different response is unclear but may be due to many factors such as different populations or treatment durations. Whereas Kappel et al found increased levels of acetyl- propionyl- and β-hydroxybutyrylcarnitine, we have observed increased levels of butyrylcarnitine[10] and, in this study, reduced plasma levels of myristoylcarnitine. When fatty acid oxidation is incomplete, surplus acyl-groups are exported from mitochondria as acylcarnitines.[16] Reduced levels of acylcarnitines therefore could reflect a larger degree of complete fatty acid oxidation. In contrast to the long-chain acylcarnitines, fasting increases the release of short-chain carnitines, including acetylcarnitine and propionylcarnitine from the hepatosplanchnic bed.[17] Several 3-hydroxy fatty acids decreased by dapagliflozin and high levels of these fatty acids are markers of fatty acid oxidation disorders.[18] Together, these results could indicate improved fatty acid oxidation and an enhanced fasting response with surplus of 2 and 4-chain carbons.

Plasma metabolomics have also been investigated in animal models treated with SGLT2 inhibitors. Treatment of obese nondiabetic mice with canagliflozin showed activation

of catabolic pathways including fatty acid oxidation and signs of inhibition of mTOR, while activation of AMPK.[19] In another study, effects of ipragliflozin on metabolites was investigated in a model of diabetic nephropathy; BTBR ob/ob mice.[20] In line with our results, they observed that SGLT2i reduced the high levels of TCA cycle intermediates in the kidney of BTBR ob/ob mice. Collectively, findings from these cross-species studies underscore metabolic effects of SGLT2i which may potentially contribute to long term clinical benefits.

SGLT-2 treatment increases hemoglobin and hematocrit. To what extent this is secondary to plasma contraction or increased red cell mass is unknown.[21] Heme, an intermediate in hemoglobin metabolism, is one of the metabolites significantly increased after dapagliflozin treatment. Increased heme levels could reflect an increased heme synthesis, but also reduced degradation of heme by heme oxygenase. Reduced plasma levels of bilirubin could indicate reduced heme degradation since carbon oxide and biliverdin which is further reduced to bilirubin are the end-products of heme oxygenase activity [22]. Regardless of the exact underlying mechanism, the increase in heme support a potential effect of SGLT2i on hematopoiesis [23].

The molecular pathways selected through our bio-informatic approach have been associated with progressive renal function loss in patients with type 2 diabetes. [24–26] The bioinformatics analysis showed that dapagliflozin treatment increased the TCA cycle activity. Expression of several TCA cycle enzymes were associated with increased eGFR indicating a positive effect on renal mitochondrial function. Increased levels of metabolites and intermediates of the TCA cycle have been associated with diabetic kidney disease and endoplasmatic reticulum stress.[27] [24] The cause of the reduced levels of TCA-cycle intermediates is not clear, but may be explained by improved mitochondrial efficacy as reflected by indications of larger degree of complete fatty acid oxidation as well as increased gluconeogenesis, which may take place both in the liver and the kidney to compensate for urinary glucose loss. Additional support of salutary effects on mitochondrial function is our finding that kynurenine was reduced while picolinic acid, a product of kynurenine metabolism, increased. This finding may indicate an increased activity of the kynurenine pathway and increased production of quinolinic acid and NAD+ that may further support improved mitochondrial function after dapagliflozin treatment. Also, the increased plasma levels of N-acetyl aspartate indicate improved mitochondrial function, specifically in the brain.[15] In line with these observations, a recent study demonstrated that a metabolite panel previously associated with reduced mitochondrial function improved after dapagliflozin in

patients with type 2 diabetes and chronic kidney disease [28]. Moreover, studies in experimental animals have shown improved mitochondrial function both in the heart and renal tubules following treatment with SGLT2i[29,30] These data together support the notion that long term renal protective effects of SGLT2i may be mediated through amelioration of mitochondrial function.

We also found the superpathway of citrulline metabolism to be associated with DKD progression and targeted/increased by dapagliflozin as evidenced by the increase in urea and reduction in glutamine. The citrulline pathway involves nitric oxide synthase as one of the key enzymes which generates citrulline from arginine in a single reaction step. Nitric oxide synthase derived nitric oxide exerts a wide array of effects and acts in the blood vessels as a potent vasodilator, exert anti-thrombotic effects and has anti-inflammatory effects [31]. Several studies have shown that SGLT2 inhibition in patients with type 2 diabetes improves endothelial function as measured by reactive hyperemia peripheral arterial tonometry and flow mediated dilation and endothelial dysfunction has been associated with accelerated renal function decline.[32–34] The exact mechanism how SGLT2 inhibition improves endothelial function are not fully understood but may involve improved glycemic and metabolic control, osmotic diuresis and changes in sodium homeostasis as a result of increased natriuresis. The current study supports a potential role for improvements in endothelial function, through the citrulline pathway as one of the potential mediators of the protective effects of SGLT2 inhibition on kidney function.

The results of the bioinformatics approach indicated a change in the glycine degradation pathway after dapagliflozin treatment. De novo creatine synthesis starts by the conversion of glycine and arginine to ornithine and guanidinoacetate by glycine amidinotransferase (GATM), and guanidinoacetate is then converted to creatine by guanidinoacetate N-methyltransferase (GAMT); a reaction that is dependent on S-adenosylmethionine as methyl donor.[35] In this study, plasma creatine levels increased, while the creatine precursor, guanidinoacetate was reduced, indicating increased GAMT activity. Alternatively, reduced GATM activity explain reduced levels of guanidinoacetate and therefore increased creatine levels must be explained by other causes than changed synthesis, such as increased dietary intake of creatine. Overall, the bioinformatics analysis suggested reduced activity of the pathway, although we cannot rule out the possibility that increased creatine levels is the result of increased GAMT activity. Therefore, the suggestion of reduced glycine degradation should be carefully interpreted.

In contrast to previous studies, this study combined metabolomics and transcriptomics to identify metabolites and pathways associated with dapagliflozin response. This approach is potentially applicable in other chronic disease settings and can be used to identify novel biomarkers for disease progression and drug efficacy and safety monitoring. The advantage is that the bio-informatic approach incorporates molecular pathway information associated with disease progression as well as biomarkers and pathway information associated with drug response thereby decreasing the likelihood of false positive findings. A limitation is that the bio-informatic approach only includes molecular features and molecular pathways which have previously been annotated and does not include novel mechanisms.

The limitations of this study include the small sample size which hampers statistical power of this study as well as the generalizability to the broader type 2 diabetes population since the studied population was a predominantly male Caucasian population. Although we compared the effect of dapagliflozin with placebo in an additional analysis, we only considered metabolites that statistically significantly changed from baseline during dapagliflozin treatment for further integration analysis since the metabolomics profile could only be determined in six patients in the placebo group. Secondly, the short follow-up precluded assessment of the effect of dapagliflozin on eGFR decline. Thirdly, the EFFECT II trial included patients with preserved kidney function whereas tissue renal transcriptomics was performed in patients with preserved or impaired renal function participating in the ERCB study. It is possible that effects of dapagliflozin on the metabolites are different in patients with different clinical characteristics, demographics and degrees of renal impairment. Hence, validation of our findings in larger studies with longer duration as well as in patients with type 2 diabetes and chronic kidney disease, such as the DAPA-CKD trial is required.[36] Unfortunately, urine was not available which could reveal a more renal specific signal. Nevertheless, our finding that metabolites related to mitochondrial function changed were in keeping with another study using urinary metabolites.[25]

In conclusion, the molecular pathways targeted by dapagliflozin and associated with diabetic kidney disease suggest that molecular processes related to energy metabolism, mitochondrial function, and endothelial function may be involved in the renal protective effects of dapagliflozin. These data confirm existing and offer novel hypothesis about the molecular effects of dapagliflozin in slowing the progression of diabetic kidney disease.

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Supplementary appendix

Supplement Table 1. Metabolites and their associated pathway which increased from baseline during dapagliflozin treatment. Geometric mean ratio from baseline represents the fold change increase from baseline within the dapagliflozin treatment group. Geometric mean ratio versus placebo represents the fold change difference between the dapagliflozin and placebo group.

Super Pathway	Sub Pathway classification	Biochemical Name	Geometric mean ratio from baseline	Geometric mean ratio vs placebo
Amino acid	Alanine and Aspartate Metabolism	N-acetylaspartate (NAA)*	1.40†	1.28‡
Amino acid	Creatine Metabolism	creatine*	1.25	1.23
Amino acid	Glycine, Serine and Threonine Metabolism	sarcosine*	1.42†	1.18
Amino acid	Guanidino and Acetamido Metabolism	guanidinosuccinate*	1.24	1.38‡
Amino acid	Histidine Metabolism	imidazole propionate*	1.44	1.97
Amino acid	Histidine Metabolism	trans-urocanate*	1.33†	1.28
Amino acid	Histidine Metabolism	formiminoglutamate*	1.32	1.50‡
Amino acid	Leucine, Isoleucine and Valine Metabolism	isobutyrylcarnitine (C4)*	1.39	1.93‡
Amino acid	Leucine, Isoleucine and	isovalerylcarnitine (C5) *	1.30†	1.21‡

	Valine Metabolism			
Amino acid	Leucine, Isoleucine and Valine Metabolism	tiglyl carnitine (C5:1-DC) *	1.23	1.42‡
Amino acid	Lysine Metabolism	N6,N6,N6-trimethyllysine*	1.29†	1.11
Amino acid	Tryptophan Metabolism	picolinate*	1.25	1.42‡
Amino acid	Urea cycle; Arginine and Proline Metabolism	argininate*	1.42	1.83‡
Amino acid	Urea cycle; Arginine and Proline Metabolism	N2,N5-diacetylornithine*	1.32†	1.55‡
Amino acid	Urea cycle; Arginine and Proline Metabolism	N-delta-acetylornithine *	1.20	1.20‡
Amino acid	Urea cycle; Arginine and Proline Metabolism	N-acetylarginine*	1.18†	1.52
Amino acid	Urea cycle; Arginine and Proline Metabolism	urea*	1.15	1.35‡
Carbohydrate	Pentose Metabolism	arabonate/xylonate	1.29†	1.45‡

Cofactors and Vitamines	Hemoglobin and Porphyrin Metabolism	heme	1.57	1.87ǂ
Cofactors and Vitamines	Tocopherol Metabolism	alpha-CEHC sulfate	1.54†	0.71
Xenobiotics	Chemical	iminodiacetate (IDA) *	1.50†	1.35
Xenobiotics	Food Component/Plant	betonicine*	1.99†	1.03

*Metabolites with a HMDB-identifier that were used for further analysis. † Metabolites that remained significant after adjustment for Δ glucose. ǂ Metabolites which significantly changed during dapagliflozin treatment compared to placebo treatment ($p<0.10$).**Supplement Table 2.** Metabolites and their associated pathway which decreased from baseline during dapagliflozin treatment. Geometric mean ratio represents the fold change decrease from baseline within the dapagliflozin treatment group. Geometric mean ratio versus placebo represents the fold change difference between the dapagliflozin and placebo group.

Supplement Table 2. Metabolites and their associated pathway which decreased from baseline during dapagliflozin treatment. Geometric mean ratio from baseline represents the fold change increase from baseline within the dapagliflozin treatment group. Geometric mean ratio versus placebo represents the fold change difference between the dapagliflozin and placebo group.

Super Pathway	Sub Pathway classification	Biochemical Name	Geometric mean ratio from baseline	Geometric mean ratio vs placebo
Amino acid	Alanine and Aspartate Metabolism	alanine*	0.90	0.87‡
Amino acid	Creatine Metabolism	guanidinoacetate*	0.87	0.81‡
Amino acid	Glutamate Metabolism	glutamine*	0.95	0.98
Amino acid	Glutathione Metabolism	5-oxoproline*	0.92	0.95
Amino acid	Leucine, Isoleucine and Valine Metabolism	4-methyl-2-oxopentanoate*	0.86	1.06
Amino acid	Leucine, Isoleucine and Valine Metabolism	3-methyl-2-oxovalerate*	0.87	1.01
Amino acid	Tryptophan Metabolism	5-bromotryptophan*	0.78†	0.85‡
Amino acid	Tryptophan Metabolism	Indolelacetate*	0.91	1.06
Amino acid	Tryptophan Metabolism	Kynurenine*	0.91	1.00
Amino acid	Tyrosine Metabolism	Thyroxine*	0.88	0.86

Amino acid	Urea cycle; Arginine and Proline Metabolism	homoarginine*	0.83†	0.92
Carbohydrate	Advanced Glycation End-product	N6-carboxymethyllysine	0.78	1.07
Carbohydrate	Fructose, Mannose and Galactose Metabolism	Mannose*	0.88	NA
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1,5-anhydroglucitol (1,5-AG) *	0.19 †	0.19‡
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	glucose*	0.88‡	0.84‡
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	glycerate*	0.91	0.89
Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	oxalate (ethanedioate) *	0.82	0.85
Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	bilirubin (E,Z or Z,E) *	0.79	0.70‡
Energy	TCA Cycle	succinate*	0.82	0.88
Energy	TCA Cycle	malate*	0.86	0.93
Energy	TCA Cycle	fumarate*	0.88	0.95
Lipid	Androgenic Steroids	andro steroid monosulfate C19H28O6S (1) *	0.73	1.17

Lipid	Androgenic Steroids	5alpha-androstan-3alpha,17beta-diol monosulfate (1)	0.75	1.34
Lipid	Androgenic Steroids	11-ketoetiocholanolone glucuronide	0.79†	1.15
Lipid	Androgenic Steroids	16a-hydroxy DHEA 3-sulfate	0.79	0.71‡
Lipid	Androgenic Steroids	epiandrosterone sulfate	0.79	1.32
Lipid	Androgenic Steroids	5alpha-androstan-3beta,17beta-diol monosulfate (2)	0.80	1.52
Lipid	Androgenic Steroids	5alpha-androstan-3beta,17beta-diol disulfate*	0.81	1.15
Lipid	Androgenic Steroids	androsterone sulfate*	0.81	1.17
Lipid	Androgenic Steroids	androstenediol (3alpha, 17alpha) monosulfate (2) *	0.83	0.99
Lipid	Androgenic Steroids	dehydroisoandrosterone sulfate (DHEA-S) *	0.90	1.20
Lipid	Corticosteroids	Cortisone*	0.92	1.04
Lipid	Diacylglycerol	linoleoyl-linolenoyl-glycerol (18:2/18:3) [1] *	0.79	0.80
Lipid	Diacylglycerol	palmitoyl-palmitoyl-glycerol (16:0/16:0) [1] *	0.83	NA
Lipid	Diacylglycerol	palmitoyl-arachidonoyl-glycerol (16:0/20:4) [2] *	0.83	0.97
Lipid	Diacylglycerol	palmitoyl-linoleoyl-glycerol (16:0/18:2) [2] *	0.83	0.97
Lipid	Endocannabinoid	N-stearoyltaurine	0.72	0.99
Lipid	Endocannabinoid	linoleoyl ethanolamide*	0.76†	0.80
Lipid	Fatty Acid Metabolism(Acyl Carnitine)	myristoylcarnitine (C14) *	0.88	NA

Lipid	Fatty Acid Metabolism(Acyl Glycine)	N-palmitoylglycine*	0.81	0.75‡
Lipid	Fatty Acid, Branched	pristanate*	0.68	0.76
Lipid	Fatty Acid, Branched	17-methylstearate*	0.86	1.05
Lipid	Fatty Acid, Dicarboxylate	octadecanedioate*	0.74†	0.84
Lipid	Fatty Acid, Dicarboxylate	tetradecanedioate*	0.78	0.79
Lipid	Fatty Acid, Dicarboxylate	Hexadecanedioate*	0.78	NA
Lipid	Fatty Acid, Dihydroxy	12,13-DiHOME*	0.44†	0.89
Lipid	Fatty Acid, Monohydroxy	3-hydroxydecanoate*	0.73†	0.83
Lipid	Fatty Acid, Monohydroxy	3-hydroxylaurate*	0.76	0.90
Lipid	Fatty Acid, Monohydroxy	13-HODE + 9-HODE	0.77	NA
Lipid	Fatty Acid, Monohydroxy	3-hydroxyoctanoate*	0.77†	0.83
Lipid	Fatty Acid, Monohydroxy	3-hydroxymyristate	0.78	0.76
Lipid	Fatty Acid, Monohydroxy	3-hydroxystearate	0.80	1.02
Lipid	Fatty Acid, Monohydroxy	2-hydroxystearate	0.86	0.88
Lipid	Lysophospholipid	1-stearoyl-GPG (18:0)	0.69	0.81
Lipid	Medium Chain Fatty Acid	10-undecenoate (11:1n1) *	0.83	1.00
Lipid	Monoacylglycerol	1-linoleoylglycerol (18:2)	0.71	0.61‡

Lipid	Monoacylglycerol	1-oleoylglycerol (18:1) *	0.72	0.63‡
Lipid	Primary Bile Acid Metabolism	glycocholate*	0.57	0.63
Lipid	Primary Bile Acid Metabolism	taurocholate*	0.59	0.61
Lipid	Primary Bile Acid Metabolism	glycochenodeoxycholate glucuronide (1)	0.69	0.69
Lipid	Progestin Steroids	5alpha-pregnan-3beta,20alpha-diol disulfate	0.77†	0.94
Lipid	Progestin Steroids	5alpha-pregnan-3beta,20beta-diol monosulfate (1)	0.79	0.91
Lipid	Progestin Steroids	5alpha-pregnan-3beta,20alpha-diol monosulfate (2)	0.81†	0.99
Lipid	Secondary Bile Acid Metabolism	ursodeoxycholate*	0.47†	0.46
Lipid	Secondary Bile Acid Metabolism	7-ketolithocholate*	0.70	0.67
Lipid	Secondary Bile Acid Metabolism	deoxycholate*	0.79†	0.92
Lipid	Secondary Bile Acid Metabolism	glycocholenate sulfate	0.82	0.86
Lipid	Sphingolipid Metabolism	sphinganine-1-phosphate*	0.75	1.06
Lipid	Sphingolipid Metabolism	sphingomyelin (d18:1/25:0, d19:0/24:1, d20:1/23:0, d19:1/24:0)	0.91	1.03
Lipid	Sphingolipid Metabolism	sphingomyelin (d18:2/24:2)	0.92	1.03
Lipid	Sterol	4-cholesten-3-one*	0.86	0.93
Nucleotide	Purine Metabolism, (Hypo)Xanthine/Inosine containing	urate*	0.81†	0.84‡

Nucleotide	Pyrimidine Metabolism, Cytidine containing	cytidine*	0.51†	NA
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylalanine *	0.77	0.69‡
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylglutamine*	0.87	0.90
Xenobiotics	Benzoate Metabolism	3-phenylpropionate (hydrocinnamate)*	0.62†	1.26
Xenobiotics	Benzoate Metabolism	2-ethylphenylsulfate	0.74†	1.12
Xenobiotics	Chemical	perfluorooctanesulfonic acid (PFOS)*	0.90	1.01
Xenobiotics	Chemical	4-hydroxychlorothalonil	0.91	0.99
Xenobiotics	Food Component/Plant	methyl glucopyranoside (alpha + beta)	0.30†	0.19‡
Xenobiotics	Food Component/Plant	eugenol sulfate	0.36	0.77
Xenobiotics	Food Component/Plant	cinnamoylglycine*	0.73	1.10
Xenobiotics	Food Component/Plant	gluconate*	0.86	0.85
Xenobiotics	Xanthine Metabolism	1,7-dimethylurate*	0.68†	0.54‡
Xenobiotics	Xanthine Metabolism	theophylline*	0.70†	0.59‡
Xenobiotics	Xanthine Metabolism	paraxanthine*	0.72	0.68

*Metabolites with a HMDB-identifier that were used for further analysis. † Metabolites that remained significant after adjustment for Δ glucose. ǂ Metabolites which significantly changed during dapagliflozin treatment compared to placebo treatment ($p<0.10$).NA, Not applicable because of a a significant ($p<0.10$) interaction between baseline metabolite concentration and treatment.